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RESEARCH PAPER

Inverse agonism of cannabinoid CB_1 receptors potentiates LiCl-induced nausea in the conditioned gaping model in rats

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BACKGROUND AND PURPOSE
Cannabinoid CB_1 receptor antagonists/inverse agonists, potentiate toxin-induced nausea and vomiting in animal models. Here, we sought to determine if this potentiated nausea was mediated by inverse agonism or neutral antagonism of the CB_1 receptor, and if the potentiated nausea would be produced by intracerebroventricular (icv) administration of an inverse agonist.

EXPERIMENTAL APPROACH
The conditioned gaping model of nausea in rats was used to compare the CB_1 receptor antagonist/inverse agonist, AM251, and the CB_1 receptor neutral antagonists, AM6527 (centrally and peripherally active) and AM6545 (peripherally active), in potentiating conditioned gaping produced by lithium chloride (LiCl) solution. The effect of icv (lateral ventricle and 4th ventricle) administration of AM251 on LiCl-induced gaping in this model was also evaluated.

KEY RESULTS
At a dose that did not produce conditioned gaping on its own, systemically administered AM251 (1.25 $mg \cdot kg^{-1}$) potentiated LiCl-induced conditioned gaping and reduced sucrose palatability; however, even doses as high as 8 $mg \cdot kg^{-1}$ of AM6545 and AM6527 neither potentiated LiCl-induced conditioned gaping nor reduced sucrose palatability. Infusions of AM251 into the lateral ventricles (1.25, 12.5 and 125 μg) or the 4th ventricle (2.5, 12.5 and 125 μg) did not potentiate LiCl-induced conditioned gaping reactions, but all doses attenuated saccharin palatability during the subsequent test.

CONCLUSIONS AND IMPLICATIONS
Inverse agonism, but not neutral antagonism, of CB_1 receptors potentiated toxin-induced nausea. This effect may be peripherally mediated or may be mediated centrally by action on CB_1 receptors, located distal to the cerebral ventricles.

Abbreviations
AP, area postrema; BBB, blood–brain barrier; DMNX, dorsal motor nucleus of the vagus; icv, intracerebroventricular; LiCl, lithium chloride; M6G, morphine 6-glucuronide; NTS, nucleus of the solitary tract; TR, Taste Reactivity

Introduction

The marijuana plant has been used for several centuries for control of nausea and vomiting (Iversen, 2000). The anti-emetic effects of cannabinoids appear to be mediated by actions at the cannabinoid CB_1 receptor (Darmani, 2001; Van Sickle *et al.*, 2001; receptor nomenclature follows Alexander *et al.*, 2009). CB_1 receptors are found in the gastrointestinal tract and its enteric nervous system (Pertwee, 2001) as well as within the emetic system of the brain (Van Sickle *et al.*, 2001; Van Sickle *et al.*, 2003) in the dorsal vagal complex, consisting of the area postrema (AP), nucleus of the solitary tract (NTS) and the dorsal motor nucleus of the vagus (DMNX) in the brainstem of rats, ferrets and the least shrew (Simoneau *et al.*, 2001; Van Sickle *et al.*, 2001; Darmani and Johnson, 2004). CB_1 receptors in the NTS are activated by centrally administered Δ^9-THC and this activation is blocked by the selective CB_1 receptor antagonists/inverse agonists, SR-141716 (Darmani and Johnson, 2004) and AM251 (Van Sickle *et al.*, 2003). Indeed, c-Fos expression induced by cisplatin in the DMNX, NTS and AP is significantly reduced by Δ^9-THC (Van Sickle *et al.*, 2001; 2003). However, recently it has been also been reported that activation of peripheral CB_1 receptors by Δ^9-THC may also play a role in the suppression of vomiting (Darmani and Johnson, 2004) and in the regulation of feeding (Gomez *et al.*, 2002; Sink *et al.*, 2009b).

Activation of CB_1 receptors clearly attenuates vomiting produced by toxins; however, the effect of activation of these receptors on nausea is less well understood. Although vomiting is well controlled by current anti-emetic therapies, the control of nausea in chemotherapy patients continues to be problematical (Andrews and Horn, 2006). Understanding the neuropharmacology of nausea so that it can be treated as effectively as vomiting would improve clinical treatment. However, the subjective nature of nausea has limited its investigation to human self-report measures. Traditionally, the potential of drugs to produce nausea has been assessed by the conditioned taste avoidance measure (see Garcia *et al.*, 1974). However, this measure has been shown to be non-selective to the nauseating effects of drugs, because drugs that do not produce nausea (and in fact even those shown to be rewarding) produce taste avoidance, and anti-emetic drugs generally do not interfere with conditioned taste avoidance (Grant, 1987; see Parker *et al.*, 2008).

Considerable recent evidence, however, suggests that conditioned gaping reactions (rapid, large amplitude opening of the mandible with retraction of the corners of the mouth) in rats represents a preclinical test for the evaluation of nausea-inducing drug effects (Parker *et al.*, 2008; Rock *et al.*, 2009; Tuerke *et al.*, 2009). Although rats are not capable of vomiting, they display characteristic conditioned gaping reactions when exposed to a flavor previously paired with a nausea-inducing treatment, such as lithium chloride (LiCl) solution (see Parker *et al.*, 2008). Unlike conditioned taste avoidance, only emetic treatments produce conditioned gaping reactions and anti-emetic drugs attenuate conditioned gaping reactions (Parker, 2003).

Cannabinoid compounds modify conditioned gaping in rats in a manner that suggests they reduce nausea as well as vomiting. Cannabinoid agonists, including Δ^9-tetrahydrocannabinol (Δ^9-THC), HU210 and URB597, interfere with the establishment of LiCl-induced conditioned gaping reactions in rats, presumably by interfering with nausea produced by LiCl (Parker and Mechoulam, 2003; Parker *et al.*, 2003; Cross-Mellor *et al.*, 2007). The interference with nausea by cannabinoid agonists is reversed by pretreatment with SR141716 (rimonabant; Parker *et al.*, 2003; Cross-Mellor *et al.*, 2007), suggesting a mechanism of action, mediated via CB_1 receptors.

Rimonabant and AM251 are not receptor neutral antagonists, instead they act as CB_1 receptor inverse agonists/antagonists (Pertwee, 2005). When administered on their own for inhibition of food intake and body weight gain, these agents produce nausea as a side effect in humans (Janero and Makriyannis, 2009). As well, they produce nausea in rats (McLaughlin *et al.*, 2005) and vomiting in the least shrew (Darmani, 2001). More recently CB_1 receptor neutral antagonists, such as AM4113, have been developed which bind to the receptor without eliciting changes in constitutive or intrinsic cellular endocannbinoid signalling. CB_1 receptor neutral antagonists (AM4113, AM6545 and AM6527) have also been shown to reduce feeding and weight gain in rats at doses comparable with Rimonabant and AM251, but without the side effect of nausea in rats or vomiting in ferrets (Chambers *et al.*, 2007; Salamone *et al.*, 2007; Sink *et al.*, 2008; Cluny *et al.*, 2010; Tam *et al.*, 2010). AM6545 also completely reversed the effects of WIN55, 212-2 in an assay of colonic motility, but had no effect on its own (Cluny *et al.*, 2010). These effects suggest that the nausea-inducing effects of compounds acting like rimonabant are mediated by their inverse agonist effects at the CB_1 receptor.

When administered at a dose that does not produce nausea or vomiting on its own, AM251 also potentiated LiCl-induced conditioned gaping reactions in rats (Parker *et al.*, 2003) and morphine

6-glucuronide (M6G) induced vomiting in ferrets (Van Sickle *et al.*, 2001; Chambers *et al.*, 2007). That is, the emetic effects of known emetic agents are potentiated in rats and ferrets pretreated with a sub-threshold dose of a CB_1 inverse agonist/antagonist. Interestingly, Chambers *et al.* (2007) recently reported that, unlike AM251, the CB_1 receptor neutral antagonist, AM4113, did not potentiate vomiting produced by M6G in the ferret, suggesting that the potentiation of toxin-induced vomiting was also mediated by inverse agonism of the CB_1 receptor. Here we present evidence that inverse agonism, but not neutral antagonism, of the CB_1 receptor also potentiated LiCl-induced nausea (at non-nauseating doses) in the conditioned gaping model in rats. The following experiments evaluated the potential of a low dose of AM251 (1.25 $mg \cdot kg^{-1}$) and a range of doses of the CB_1 receptor neutral antagonists, AM6545 [with limited penetration across the blood–brain barrier (BBB); see Makriyannis *et al.*, 2009; Cluny *et al.*, 2010; Tam *et al.*, 2010] and AM6527 (which crosses the BBB; see Sink *et al.*, 2009a), to potentiate LiCl-induced nausea in rats.

As there is evidence that both central and peripheral CB_1 receptors may be involved in the control of vomiting responses in emetic species (van Sickle *et al.*, 2001; Darmani and Johnson, 2004), it is unclear whether the potentiation of LiCl-induced nausea by systemically administered CB_1 receptor inverse agonists/antagonists in rats is centrally or peripherally mediated. Therefore, additional experiments evaluated the effect of intracerebroventricular (icv) injections of AM251 on LiCl-induced nausea in rats. In separate experiments, AM251 was infused into the lateral ventricles (primarily targeting forebrain structures) and to the 4th ventricles (primarily targeting brainstem structures) and the effect on LiCl-induced conditioned gaping was measured.

Methods

Animals

All animal care and experimental procedures were approved by the Animal Care Committees of Wilfrid Laurier University and the University of Guelph and adhered to the guidelines of the Canadian Council of Animal Care. Animals were maintained on *ad libitum* food (Highland Rat Chow [8640]) and water throughout the experiments. We used male Sprague Dawley rats (Charles River Lab, St Constant, Quebec) weighing from 300 to 380 g on the day of conditioning. The rats were individually housed in Plexiglas cages (48 × 26 × 20 cm) in the colony room at an ambient temperature of 21°C with a 12/12 reverse light/dark schedule (lights off at 7 am). All experimental manipulations were conducted during the dark phase of the cycle. Rats were provided with two clean paper towels (replenished during weekly cage changes) and a soft plastic container that was 14 cm long and 12 cm in diameter that remained in the home cage.

Intraoral cannulation surgery

All rats were implanted with intraoral cannula for delivery of the 0.1% saccharin solution directly to the oral cavity. On the day of surgery, the rats were injected with an antibiotic (Derapin: 100 $mg \cdot kg^{-1}$, sc; Ayerst) 30 min prior to being anaesthetized with isoflurane (4–5% induction, 1.5% maintenance in O_2). Once anaesthetized a 2 cm^2 section of skin was shaved at the back of the neck at the level of the scapula. The skin was prepared by cleaning with soap (Bactistat; Ecolab, St. Paul, MN, USA) and wiping with 70% isopropyl alcohol followed by 7% Betadine solution (Purdue Products L.P., Stamford, CT, USA). The rat was then administered a 5 $mg \cdot kg^{-1}$ injection (ip) of the anti-inflammatory/analgesic drug carprofen (Rimadyl; Pfizer Canada Inc., Kirkland, QC, Canada). A thin-walled 15 G stainless steel needle was inserted at the shaved area on the neck, directed subcutaneously around the ear and brought out behind the first molar inside the mouth. A 10 cm length of Intra Medic PE90 tubing (Clay Adams Brand; Becton Dickinson and Co., Sparks, MD, USA) with an inner diameter (I.D.) of 0.86 mm and an outer diameter (O.D.) of 1.27 mm was then inserted through the needle after which the needle was removed. Betadine (10%) was applied to the puncture site and two elastic discs (2 cm^2) were placed over the exposed end of the tubing and drawn to the skin at the back of the neck for the purpose of stabilizing the cannula. The cannula was held secure in the oral cavity by a 6 mm disc of polypropylene mesh (297 micron; Small Parts Inc., Miramar, FL, USA) secured behind the heat flanged intraoral opening. Each cannula was constructed prior to surgery and kept in a cold sterilent solution (Germiphene Corporation, Brantford, ON, Canada). Rats were returned to their home cage immediately following surgery and food pellets were loosely provided in the cage. For 3 days following surgery indices of recovery were assessed (body weight, facial swelling, activity, etc.) and the cannula was flushed daily with an oral cleansing solution (Nolvadent; Ayerst, Fort Dodge, Iowa, USA).

Stereotaxic guide cannula surgery

For the studies of central effects of AM251, rats had a unilateral indwelling guide cannula surgically implanted in the lateral ventricle (Experiment 3a) or

the 4th ventricle (Experiment 3b). They were anaesthetized with isoflurane gas and prepared for surgery by shaving a strip of skin between the ears (2.5–3 cm long), cleaning the skin and injecting carprofen (5 mg·kg^{-1}; ip) and a local anaesthetic (0.1 mL; sc; Marcaine; Hospira, Montreal, QC, Canada) on either side of the skull. They were then stabilized in the flat skull position (according to Paxinos and Watson, 1998) in the stereotaxic frame and the skull was exposed. In Experiment 3a, the stainless steel guide cannula (22G, 8 mm below pedestal) was lowered into either the left or right lateral ventricle (counterbalanced among groups) using the following coordinates from Bregma: –1.0 mm AP, ±1.4 mm LM, –3.6 mm DV (Erb *et al.*, 2003). In Experiment 3b, the guide cannula (22 G, 11 mm below pedestal) was lowered into the 4th ventricle in the right or left hemisphere (counterbalanced among groups) using the following coordinates from interaural zero: AP –3.5; LM ± 1.4; DV –6.2 (Blevins *et al.*, 2004). The guide cannula was stabilized by four screws secured in the skull and dental cement. Once the dental cement had hardened, a stainless steel obdurator was inserted in the guide cannula to maintain patency. Immediately following removal from the stereotaxic frame, the rats were surgically implanted with an intraoral cannula then placed in a heated recovery area and monitored until they were ambulatory, at which time they were returned to the colony room. The rats were allowed to recover for 5 days following surgery and were monitored daily as previously described.

Histology

Verification of lateral ventricle cannula placement was conducted 3 days following surgery by injecting 2 µL angiotensin II (50 ng/2 µL) at a rate of 2 µL·min^{-1}. If the rat drank from a water bottle within 1 min of the infusion and sustained drinking for 2–3 min the cannula was considered to be accurately placed (Erb *et al.*, 2003), otherwise it was removed from further testing.

Verification of cannula placement into the 4th ventricle was evaluated by histological evaluation of tissue. Rats were deeply anaesthetized using an 85 mg·kg^{-1} injection of Euthansol (Intervet Canada Corp., Kirkland, QC, Canada) followed by transcardial perfusion with phosphate buffered saline (0.1 M) and 4% formalin. The brains were removed and stored at 4°C in 4% formalin solution for 24–48 h after which they were placed in a 20% sucrose solution overnight at room temperature. The brains were then sliced in 60 µm sections using a CM1850 Leica cryostat and relevant sections were mounted on glass microscope slides. The tissue was stained with cresyl violet 24 h later and examined for accurate cannula placement using a Leica MZ6 Stereomicroscope with a Leica DFC420 Digital Camera and Leica Application Suite software. Data from any rat with improper cannula placement were excluded from the behavioural analyses.

Apparatus

Conditioning and testing was conducted in a square Plexiglas 'Taste Reactivity' (TR) chamber (26.5 × 26.5 × 12 cm) placed on a glass-topped table with a mirror set at 45° below the glass top to facilitate videotaping the ventral surface of the rat while in the chamber. The rat's cannula was attached to the infusion pump (KDS100; KD Scientific Inc, Holliston, MA, USA) using a length of PE160 tubing fitted over the intraoral cannula that ran through a hole in the top of the chamber and connected to the pump. Orofacial responses were captured directly to the computer using the Roxio Videowave Premiere Suite 8 video capture program and a Sony DCR-HC28 Handycam camera.

For central administration of solutions, microinfusions were conducted using a 28 gauge injector that extended 1 mm beyond the guide cannula tip and was attached to a microinfusion pump (KDS101; KD Scientific Inc, Holliston, MA, USA) using Tygon tubing with a 0.1905 mm ID (Cole Parmer, Vernon Hills, IL, USA).

Procedures

Experiment 1: Potential of systemic AM251 to potentiate LiCl-induced conditioned gaping. The TR procedure was similar in all experiments except for the drugs delivered. Three days after recovering from the intraoral cannulation surgery, the rats received an adaptation trial; they were individually placed in the TR chamber with their cannula attached to an infusion pump for fluid delivery. Water was infused into their intraoral cannula over a period of 2 min at the rate of 1 mL·min^{-1} after which they were returned to their home cage. The rats received a conditioning trial 24 h after the adaptation trial. Prior to the conditioning trial, the rats were randomly assigned to groups on the basis of treatment drug (AM251/vehicle) and conditioning drug (LiCl/saline) as follows: AM251/LiCl (n = 7), AM251/Saline (n = 7), Vehicle/LiCl (n = 8) and Vehicle/Saline (n = 8). Thirty min prior to the conditioning trial, the rats were injected ip with either 1.25 mg·kg^{-1} AM251 or vehicle according to group assignment. They were then placed in the TR chamber and were intraorally infused with 0.1% saccharin for 2 min at a rate of 1 mL·min^{-1}. Immediately following the saccharin infusion, the rats were injected with either 20 mL·kg^{-1} of LiCl or saline according to group assignment and returned to their home cage. The

animals were given a second adaptation trial with a 2 min intraoral infusion of water, 48 h following the conditioning trial and 24 h prior to a drug-free TR test trial. The rats were placed in the TR chamber and infused with 0.1% saccharin solution over a period of 2 min (1 mL·min^{-1}) while their orofacial reactions were recorded from the mirror set at a 45° angle beneath the glass floor of the chamber.

Experiment 2: Potential of CB1 receptor neutral antagonists to potentiate LiCl-induced conditioned gaping. The rats were treated as in Experiment 1 except as indicated. In Experiment 2a, the rats were assigned to groups on the basis of treatment drug (AM6545/Vehicle) and conditioning drug (LiCl/Saline) as such: Vehicle/LiCl (n = 8), Vehicle/Saline (n = 8), 1.25 mg·kg^{-1} AM6545/LiCl (n = 8), 1.25 mg·kg^{-1} AM6545/Saline (n = 8), 2.5 mg·kg^{-1} AM6545/LiCl (n = 8), 2.5 mg·kg^{-1} AM6545/Saline (n = 9), 8 mg·kg^{-1} AM6545/LiCl (n = 8), 8 mg·kg^{-1} AM6545/Saline (n = 8). In Experiment 2b, the rats were assigned to groups on the basis of treatment drug (AM6527/Vehicle) and conditioning drug (LiCl/Saline) as such: Vehicle/LiCl (n = 8), Vehicle/Saline (n = 6), 8 mg·kg^{-1} AM6527/LiCl (n = 8), 8 mg·kg^{-1} AM6527/Saline (n = 8). Twenty-four hours after the adaptation trial, on the conditioning trial, the rats were injected with the treatment drug 30 min prior to receiving a 2 min intraoral infusion of 0.1% saccharin solution (1 mL·min^{-1}) and were immediately injected with conditioning drug. Seventy-two hours later (24 h after the second adaptation trial), the rats received a drug-free 2 min TR test with 0.1% saccharin solution.

Experiment 3: Potential of icv AM251 given into the lateral ventricle or the 4th ventricle to potentiate LiCl-induced conditioned gaping. Experiment 3 was conducted as Experiment 1, except AM251 or vehicle was delivered icv to either the lateral or the 4th ventricle. All rats had a unilateral indwelling cannula surgically implanted in the lateral ventricle (Experiment 3a) or the 4th ventricle (Experiment 3b). The TR adaptation trial occurred on the sixth day following surgery and was conducted as in Experiment 1. The conditioning trial occurred 24 h after the adaptation trial. In Experiment 3a (lateral ventricle placement), the rats were randomly assigned to groups on the basis of treatment drug (Vehicle, 1.25 µg AM251, 12.5 µg AM251 or 125 µg AM251) and conditioning drug (LiCl or saline) as follows: Vehicle/LiCl (n = 7), Vehicle/Saline (n = 7), 1.25 µg AM251/LiCl (n = 7), 1.25 µg AM251/Saline (n = 7), 12.5 µg AM251/LiCl (n = 7), 12.5 µg AM251/Saline (n = 8), 125 µg AM251/LiCl (n = 6), 125 µg AM251/Saline (n = 6). In Experiment 3b (4th ventricle placement), the rats were assigned to the following groups: Vehicle/LiCl (n = 8), Vehicle Saline (n = 6), 2.5 µg AM251/LiCl (n = 8), 2.5 µg AM251/Saline (n = 7), 12.5 µg AM251/LiCl (n = 6) and 12.5 µg AM251/Saline (n = 5). In Experiment 3b, an additional group was also administered 125 µg AM251/LiCl (n = 4) and was compared with group Vehicle/LiCl. All n-values refer to rats with correct cannula placements.

On the conditioning trial of both experiments, the obdurator was removed from the guide cannula and while the rat was restrained (wrapped in a towel) a 28 G injector was inserted and 2 uL of the treatment drug was microinfused at a rate of 2 µL·min^{-1}. Following the infusion, the injector was left in place for 1 min before removal and replacement of the obdurator. Thirty min later, the rats were placed in the TR chamber and intraorally infused with 2 mL of 0.1% saccharin solution at the rate of 1 mL·min^{-1} and their orofacial reactions were videotaped. Forty-eight hours later, the rats received an adaptation trial followed 24 h later by the drug-free test trial during which they were intraorally infused with 0.1% saccharin solution and their orofacial reactions videorecorded.

Behavioural measures

The video recordings were scored using the Observer (Noldus Information Technology, Sterling, VA) event recording program for the following behaviours: The number of *gaping reactions* (rapid, large amplitude opening of the mandible with retraction of the corners of the mouth) was counted during the 2 min sessions. The number of seconds that the rats displayed *hedonic reactions* of mouth movements (movement of the lower mandible without opening the mouth) and tongue protrusions (extensions of the tongue out of the mouth) was also measured and the total of the two measures was used as the hedonic score.

Data analysis

In each experiment, the rats did not display gaping reactions during the conditioning trial. Therefore, the number of gaping reactions during the test trials only were entered into a between factors analysis of variance (ANOVA), with the factors of treatment drug and conditioning drug. Subsequent Bonferroni *post hoc* comparison or planned comparison tests were used as appropriate. The hedonic reaction scores were entered into mixed factors ANOVAs, with treatment drug, conditioning drug and trial (conditioning trial/ test trial) as factors. When appropriate, separate, two factor, between groups, ANOVAs were then conducted for the conditioning trial and the test trial.

Materials

Lithium chloride and angiotensin II were obtained from Sigma-Aldrich Canada, Toronto, ON, Canada; Isoflurane from Benson Medical Industries Inc. Toronto, ON Canada and 2-hydroxypropyl-β-cyclodextrin (2-HPBCD) from ONBIO Inc, Richmond Hill, ON, Canada. The AM compounds (AM251, AM6545 and AM6527) were synthesized at the Center for Drug Discovery, Northwestern University. The stainless steel guide cannula was from Plastics One Inc., Roanoke, VA, United States.

Lithium chloride was prepared in a 0.15 M solution with sterile water and was administered i.p. in a volume of 20 mL·kg^{-1} (127.2 mg·kg^{-1}). AM251, AM6545 and AM6527 were prepared (1 mg·mL^{-1}) in a vehicle of 45% 2-HPBCD in sterile water.

Results

Experiment 1: Effects of systemic AM251 on LiCl-induced conditioned gaping

When administered ip, a low dose of AM251 potentiated LiCl-induced conditioned gaping, as has been reported with rimonbant (Parker *et al.*, 2003). The upper section in Figure 1 presents the mean number of gaping reactions displayed by the various groups in Experiment 1 during the conditioning trial and during the test trial. As is apparent, the rats did not display gaping reactions during the conditioning trial. The mean number of gaping reactions during the test trial was entered into a 2 × 2 ANOVA which revealed significant effects of treatment drug, F (1, 26) = 7.8, $P < 0.01$, conditioning drug, F (1, 26) = 56.7; $P < 0.001$ and a significant (treatment drug × conditioning drug) interaction, F (1, 26) = 5.0; $P < 0.05$. Subsequent planned comparison tests revealed that the groups Veh-LiCl and AM251-LiCl displayed more gaping reactions than the groups Veh-Saline or AM251-Saline ($P < 0.01$); however, the group AM251-LiCl also displayed significantly more gaping reactions than the group Veh-LiCl ($P < 0.01$).

Systemic administration of AM251 also modified hedonic reactions elicited by saccharin solution during both conditioning and testing. The bottom section of Figure 1 presents the mean number of seconds that the rats displayed hedonic reactions during the conditioning trial and the test trial of Experiment 1. The mixed factors ANOVA revealed significant effects of treatment drug, F (1, 26) = 16.5; $P < 0.001$, conditioning drug, F (1, 26) = 4.5; $P < 0.05$, and a (treatment drug × conditioning drug) interaction, F (1, 26) = 5.3; $P < 0.05$, as well as trial, F (1, 26) = 24.5; $P < 0.001$, (conditioning drug × trial), F (1, 26) = 13.8; $P < 0.001$ and a (treatment drug × conditioning drug × trial) interaction, F (1, 26) = 7.0; $P < 0.025$. On the conditioning trial, a 2 × 2 ANOVA revealed only a significant effect of treatment drug, F (1, 26) = 19.8; $P < 0.001$, with rats treated with AM251 displaying fewer hedonic reactions than rats treated with vehicle (replicating Jarrett *et al.*, 2007). On the test trial, a 2 × 2 ANOVA revealed a significant effect of treatment drug, F (1, 26) = 7.5; $P < 0.01$, conditioning drug, F (1, 26) = 10.6; $P < 0.01$ and an (treatment drug × conditioning drug) interaction, F (1, 26) = 9.2; $P < 0.01$. Planned comparison tests of the data for the test trial revealed that the group Veh-Saline displayed more hedonic reactions than any other group (Ps < 0.01) and the remaining groups did not differ from one another.

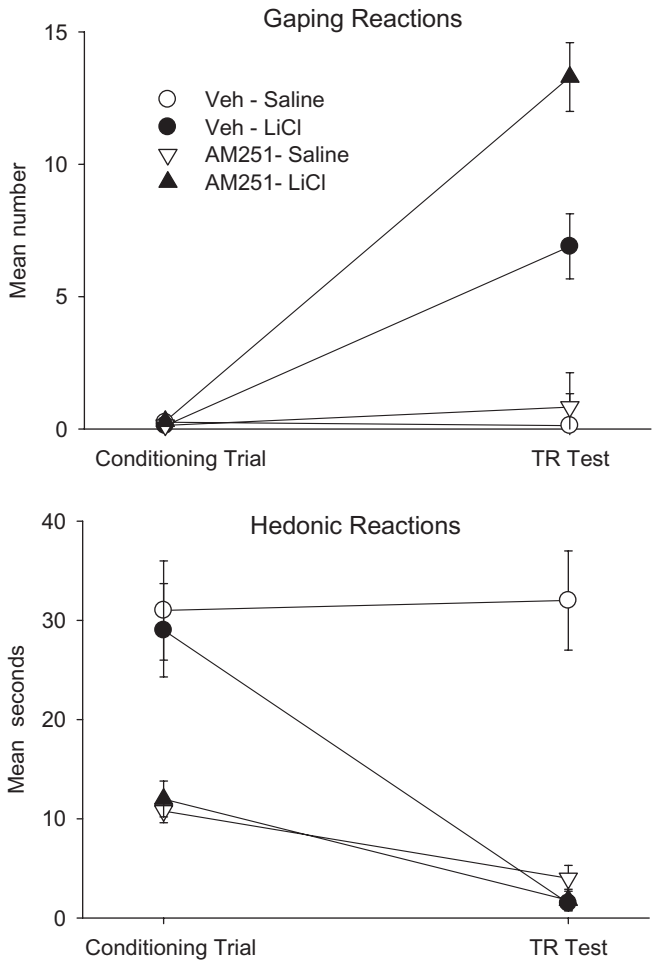

Figure 1

Mean (±SEM) number of gaping reactions (upper section) and mean (±SEM) seconds spent displaying hedonic reactions (tongue protrusions + mouth movements) during the conditioning trial and the drug-free TR test trial by rats injected (ip) with vehicle or 1.25 mg·kg^{-1} AM251 30 min prior to a saccharin–LiCl or saccharin–saline pairing. LiCl, lithium chloride; TR, Taste Reactivity.

Experiment 2: Effects of CB_1 receptor neutral antagonists on LiCl-induced conditioned gaping

Systemic administration of neither the CB_1 receptor neutral antagonist, AM6545 (peripherally restricted) nor AM6527 (crosses BBB), potentiated LiCl-induced conditioned gaping. These two compounds did not suppress hedonic reactions – during conditioning or during the subsequent drug-free test.

Experiment 2a. Even at a dose as high as 8 $mg \cdot kg^{-1}$, which suppresses feeding (Cluny *et al.*, 2010; Tam *et al.*, 2010), the peripherally restricted CB_1 neutral antagonist, AM6545, did not potentiate LiCl-induced conditioned gaping in rats. The upper section of Figure 2 presents the mean number of gaping reactions displayed by the rats in the various groups on the conditioning trial and on the test trial. On the test trial, the 4 × 2 ANOVA revealed only a significant effect of conditioning drug, F (1, 57) = 23.6; $P < 0.001$; LiCl produced conditioned gaping, but the strength of the response did not significantly differ among the treatment drug groups.

Also, unlike AM251, AM6545 neither unconditionally nor conditionally suppressed hedonic reactions elicited by saccharin solution. The lower section of Figure 2 presents the mean seconds of hedonic reactions displayed by the various groups on the conditioning trial and on the TR test trial. The mixed factors ANOVA revealed only a significant effect of trial, F (1, 57) = 22.5; $P < 0.001$ and an (conditioning drug × trial) interaction, F (1, 57) = 7.2; $P = 0.01$. Separate 4 × 2 between groups ANOVAs for each trial revealed only a significant effect of conditioning drug on the test trial, F (1, 57) = 28.6; $P < 0.001$. Rats showed suppressed hedonic reactions to LiCl-paired saccharin on the test trial, but the strength of the suppression was not affected by treatment with AM6545.

Experiment 2b. The centrally active CB_1 receptor neutral antagonist, AM6527 (8 $mg \cdot kg^{-1}$), neither potentiated LiCl-induced conditioned gaping nor suppressed hedonic reactions elicited by saccharin solution during the conditioning or test trials. The upper section of Figure 3 presents the mean number of gaping reactions displayed by the rats in the various groups on the conditioning trial and on the TR test trial. A 2 × 2 between groups ANOVA for the test trial revealed only a significant effect of conditioning drug, F (1, 26) = 14.5; $P < 0.001$; that is, LiCl produced conditioned gaping that did not significantly differ among treatment groups. The lower section of Figure 3 presents the mean seconds of hedonic reactions displayed by the various groups on the conditioning trial and TR test trial. The mixed factors ANOVA revealed only a significant (conditioning drug × trial) interaction, F (1, 26) = 6.6; $P = 0.016$. Planned comparison tests revealed that regardless of treatment condition, the LiCl-conditioned groups displayed fewer hedonic reactions on the test trial than on the conditioning trial ($P < 0.01$), while the saline-conditioned groups did not differ in hedonic reactions displayed during conditioning or testing.

Experiment 3: Effects of AM251, given icv, to the lateral or the 4th ventricle on LiCl-induced conditioned gaping

Whether administered to the lateral ventricle or to the 4th ventricle, AM251 did not potentiate LiCl-induced conditioned gaping, even though it did modify saccharin palatability. The upper sections of Figures 4 and 5 present the mean number of gaping reactions displayed by the various groups on the conditioning trial and the test trial of Experiments 3a and 3b when AM251 or vehicle was delivered to the lateral ventricle or the 4th ventricle, respectively, prior to conditioning. In Experiment 3a (lateral ventricle) on the test trial, the 4 × 2 between groups ANOVA of gaping reactions revealed only a significant conditioning drug effect, F (1, 47) = 25.6; $P < 0.001$. Likewise, the 3 × 2 between groups ANOVA of gaping reactions on the test trial in Experiment 3b (4th ventricle) revealed a significant effect of conditioning drug, F (1, 34) = 27.7; $P < 0.001$. Finally in Experiment 3b, the mean number of gaping reactions displayed by the additional four rats given 125 µg AM251/LiCl (10.2 ± 4.3) did not significantly differ from the group Vehicle/LiCl (12.8 ± 4.0). In both Experiments 3a and 3b, LiCl did induce conditioned gaping in rats, but the treatment drug did not modify the strength of the gaping reactions.

Central administration of AM251 to either the lateral or 4th ventricle did, however, conditionally attenuate the palatability of saccharin reflected in reduced hedonic reactions during the test trial, as can be seen in the lower sections of Figures 4 and 5 respectively. Furthermore, when delivered to the lateral ventricle, but not to the 4th ventricle, AM251 also suppressed hedonic reactions unconditionally during the conditioning trial. In Experiment 3a (lateral ventricle), the mixed factors ANOVA of hedonic reactions revealed significant effects of treatment drug, F (3, 47) = 6.9; $P < 0.001$, trial, F (1, 47) = 56.7; $P < 0.001$ (conditioning drug × trial), F (1, 47) = 12.5; $P < 0.001$ and an (treatment drug × conditioning drug × trial) interaction, F (3, 47) = 2.8; $P < 0.05$. A separate 4 × 2 ANOVA for the conditioning trial in Experiment 3a (lateral ventricles) revealed a significant effect of pretreatment condition, F (3, 47) = 4.1; $P < 0.025$; Bonferroni *post hoc* pairwise

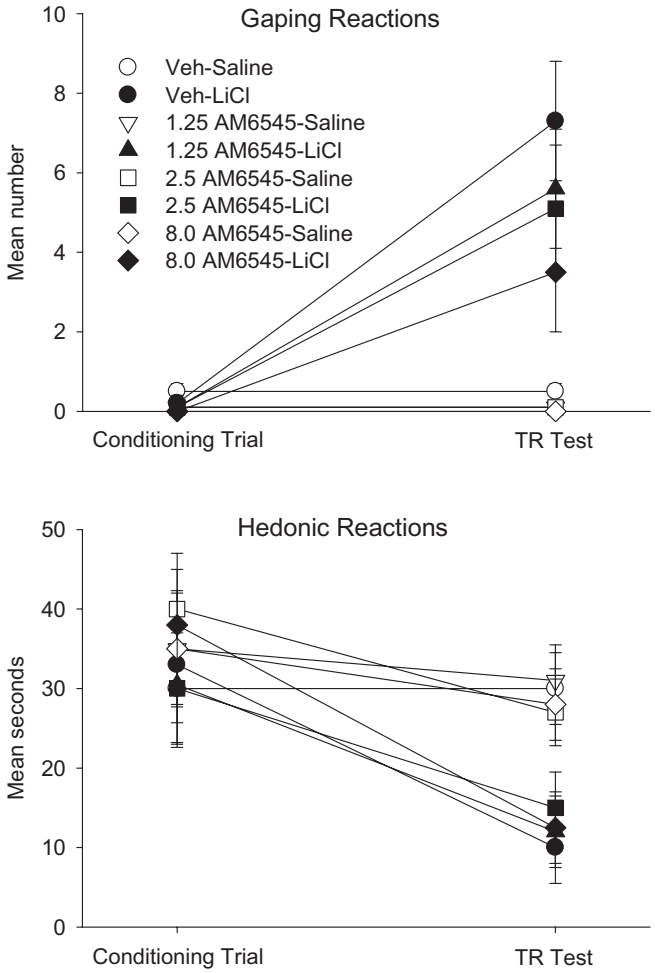

Figure 2

Mean (±SEM) number of gaping reactions (upper section) and mean (±SEM) seconds spent displaying hedonic reactions (lower section) during the conditioning trial and the drug-free TR test trial by rats injected (ip) with vehicle, 1.25, 2.5 or 8 mg·kg^{-1} AM6545 30 min prior to a saccharin–LiCl or saccharin–saline pairing. LiCl, lithium chloride; TR, Taste Reactivity.

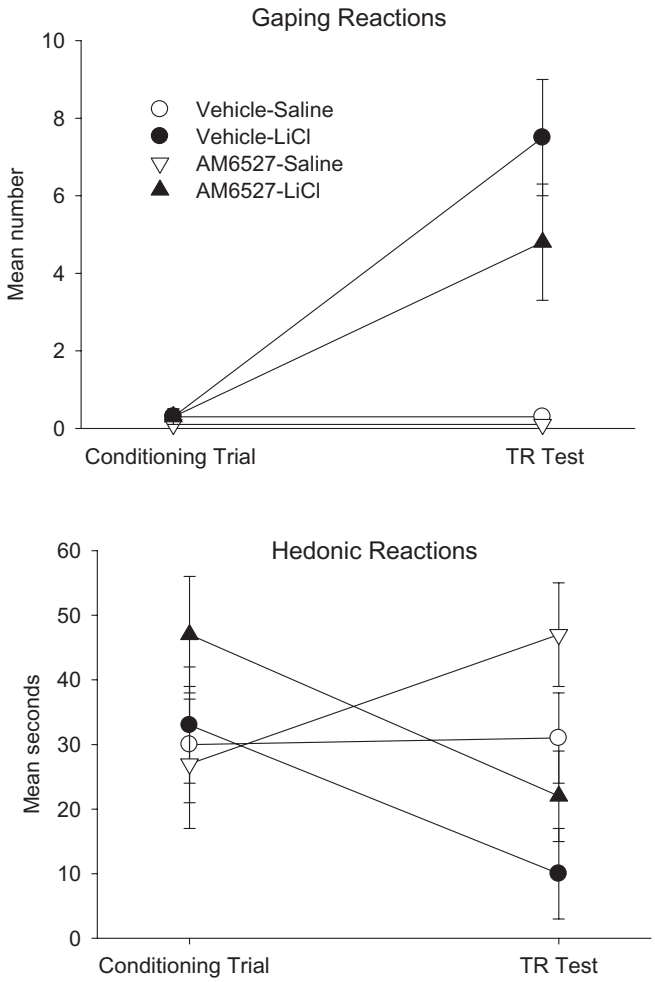

Figure 3

Mean (±SEM) number of gaping reactions (upper section) and mean (±SEM) seconds spent displaying hedonic reactions (lower section) during the conditioning trial and the drug-free TR test trial by rats injected (ip) with vehicle or 8 mg·kg^{-1} AM6527 30 min prior to a saccharin–LiCl or saccharin–saline pairing. LiCl, lithium chloride; TR, Taste Reactivity.

comparison tests revealed only that rats pretreated with vehicle displayed significantly more hedonic reactions than did the rats pretreated with any dose of AM251 ($P < 0.05$). The 4 by 2 ANOVA for the test trial revealed significant effects of treatment drug, F (3, 47) = 7.0; $P < 0.001$, conditioning drug, F (1, 47) = 9.7; $P < 0.01$ and an (treatment drug × conditioning drug) interaction, F (3, 47) = 5.7; $P < 0.01$. Bonferroni *post hoc* pairwise comparison tests revealed that the group Vehicle-Saline displayed significantly more ($P < 0.01$) hedonic reactions than any other group, which did not differ from each other. In Experiment 3b (4th ventricle), the mixed factors ANOVA of hedonic reactions revealed significant effects of conditioning drug, F (1, 34) = 5.5; $P <$ 0.025, trial, F (1, 34) = 39.7; $P < 0.001$, (conditioning drug × trial), F (1, 34) = 4.8; $P < 0.05$ and (treatment drug × conditioning drug × trial), F (2, 34) = 3.4; $P <$ 0.05. Separate 3 by 2 ANOVAs of the hedonic reactions for the conditioning and test trials, revealed only significant effects on the TR test trial of treatment drug, F (2, 34) = 7.5; $P < 0.01$, conditioning drug, F (1, 34) = 19.4; $P < 0.01$ and an (treatment drug × conditioning drug) interaction, F (2, 34) = 7.0; $P < 0.01$. Bonnferoni *post hoc* pairwise comparison tests revealed that on the test trial the group Vehicle-Saline displayed significantly more hedonic reactions than any other group ($P < 0.01$), but the other groups did not significantly differ from one another.

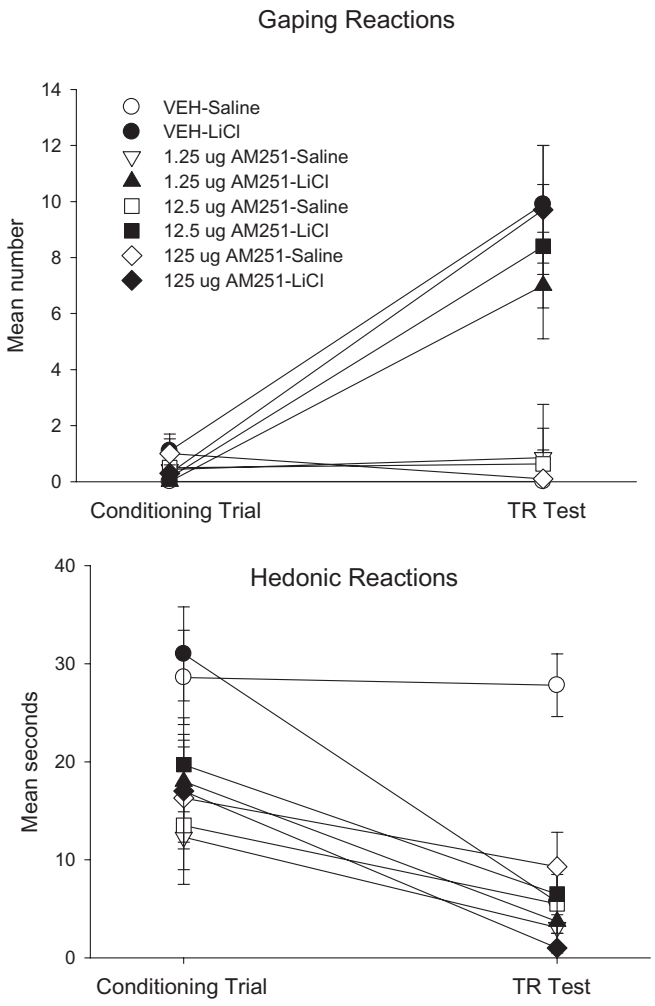

Figure 4

Mean (±SEM) number of gaping reactions (upper section) and mean (±SEM) seconds spent displaying hedonic reactions (lower section) during the conditioning trial and the drug-free TR test trial by rats injected (icv) with vehicle, 1.25, 12.5 or 125 µg AM251 into the lateral ventricles 30 min prior to a saccharin–LiCl or saccharin–saline pairing. LiCl, lithium chloride; TR, Taste Reactivity.

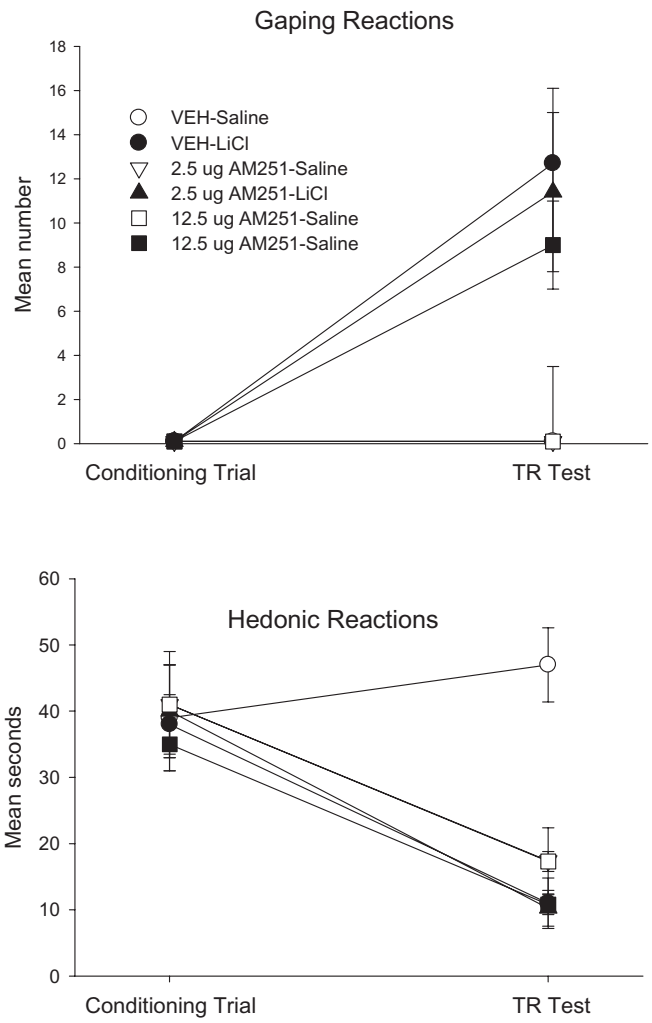

Figure 5

Mean (±SEM) number of gaping reactions (upper section) and mean (±SEM) seconds spent displaying hedonic reactions (lower section) during the conditioning trial and the drug-free TR test trial by rats injected (icv) with vehicle, 2.5 or 12.5 µg AM251 into the fourth ventricle 30 min prior to a saccharin–LiCl or saccharin–saline pairing. LiCl, lithium chloride; TR, Taste Reactivity.

Discussion

Systemic administration of a low dose (1.25 mg·kg^{-1}) of the CB_1 antagonist/inverse agonist, AM251, that does not produce gaping on its own (McLaughlin *et al.*, 2005), potentiated conditioned gaping reactions elicited by a LiCl-paired saccharin solution in rats, as has been reported with another inverse agonist, rimonabant (Parker *et al.*, 2003). On the other hand, systemic injections of much higher doses (up to 8 mg·kg^{-1}) of the CB_1 antagonists with no inverse agonist action, AM6545 (peripherally restricted; see Makriyannis *et al.*, 2009; Cluny *et al.*, 2010; Tam *et al.*, 2010) and AM6527 (peripherally and centrally active; Sink *et al.*, 2009a), did not potentiate LiCl- induced conditioned gaping in rats. At the same range of doses both AM6545 (Makriyannis *et al.*, 2009; Cluny *et al.*, 2010) and AM6527 (Sink *et al.*, 2009a) have been shown to suppress feeding and reduce body weight in rats.

Our results suggest that in the presence of a toxin, inverse agonism of the CB_1 receptor, enhances the nausea produced by that toxin. This effect on LiCl-induced nausea may be peripherally mediated, because icv administration of AM251 (even at a dose 1/10th that of the peripheral dose) either to the lateral ventricle or the 4th ventricle did not produce a similar potentiation of conditioned gaping reactions. Recent evidence suggests that differential effects on feeding occur with

administration of a melanocortin antagonist (Fan *et al.*, 2004) or an oxytocin antagonist (Blevins *et al.*, 2004) into the 3rd or the 4th ventricles. As brainstem sites have been shown to be important for the emetic reaction in ferrets (e.g.,Van Sickle *et al.*, 2001) and LiCl-induced conditioned gaping (Eckel and Ossenkopp, 1996) and taste avoidance (Ritter *et al.*, 1980) in rats, the 4th ventricle injections were expected to produce a greater effect on LiCl-induced potentiated nausea than the lateral ventricle injections. However, neither infusions of AM251, at doses as high as 1/10th the peripheral dose, to the lateral or the 4th ventricle produced enhancement of LiCl-induced conditioned gaping reactions. Interestingly, when LiCl is administered to the lateral ventricles in rats, it is also ineffective in producing conditioned taste avoidance (Smith, 1980).

Given that 5-HT_3 receptor antagonists, such as ondansetron, are the most commonly employed anti-emetic agents to treat the side effects of emesis and nausea in cancer chemotherapy, and that ondansetron blocks LiCl-induced conditioned gaping in rats (Limebeer and Parker, 2000), it is conceivable that the interaction of peripheral CB_1 receptor inverse agonism and 5-HT_3 receptor agonism mediated the potentiated aversion. Cannabinoid agonists have been reported to inhibit the activity of 5-HT_3 receptors on the rat nodose ganglion (Fan, 1995), an effect that may be mediated by allosteric modulation of the 5-HT_3 receptor by the cannabinoid agonist (Barann *et al.*, 2002). CB_1 receptors are expressed in the nodose ganglion and largely transported to peripheral terminals of the vagus (Partosoedarso *et al.*, 2003), providing another potential site of interaction between 5-HT_3 and CB_1 receptors in the regulation of nausea and vomiting. It has also been suggested that cannabinoids may act on the interneurons synapsing with the vagal motor neurons (Hornby and Prouty, 2004). The vagal afferents project to the NTS, clearly a site of anti-emetic action of CB_1 receptor agonists (Van Sickle *et al.*, 2003), and from here information is disseminated to higher regions of the brain including the parabrachial nucleus, hypothalamus, amygdala and insular cortex (Andrews and Sanger, 2002). The generation of the sensation of nausea is most likely cortically driven (insular cortex; Penfield and Faulk, 1955), but the present data suggest that the initial site of action of the CB_1 receptor inverse agonist in enhancing LiCl-induced nausea may be in the periphery. This effect would be expected to be blocked by subdiaphramatic vagotomy. Future studies aimed at evaluating the effect of systemic pretreatment with AM251 on LiCl-induced potentiated conditioned gaping in vagotomized rats would be valuable in testing this hypothesis.

On the other hand, it is also conceivable that the action of AM251 on central CB_1 receptors located distal from the cerebral ventricles is responsible for the potentiation of LiCl-induced nausea. One candidate region is the insular cortex and there is considerable evidence that the insular cortex regulates the sensation of nausea. Electrical stimulation of the insular cortex produces vomiting in cats (Kaada, 1951) and humans (Catenoix *et al.*, 2008), as well as a sensation of nausea in humans (Fiol *et al.*, 1988; Penfield and Faulk, 1955). Contreras *et al.* (2007) recently reported that inactivation of the interoceptive insula attenuated LiCl-induced malaise in rats as measured by flattened 'lying on belly' postures (Parker *et al.*, 1984), which was also attenuated by AP lesions (Bernstein *et al.*, 1992) and ondansetron pretreatment (unpublished observations). Finally, ablation of the insular cortex (Kiefer and Orr, 1992), but not the amygdala (Rana and Parker, 2008), prevents LiCl-induced conditioned gaping reactions in rats (Kiefer and Orr, 1992). Indeed, recent human evidence indicates that the insular cortex, but not the amygdala, is activated by scenes of disgusting food (Calder *et al.*, 2007).

Depletion of forebrain 5-HT by 5,7-dihydroxytryptamine (5,7-DHT) lesions of the dorsal and median raphe nuclei completely blocks LiCl-induced conditioned gaping reactions in rats (Limebeer *et al.*, 2004). Therefore, it is likely that the action of 5-HT in forebrain regions, most likely the insular cortex, is necessary for the sensation of nausea. CB_1 receptors are found within this region (Tsou *et al.*, 1998) and they are co-expressed with 5-HT_3 receptors in the insular cortex (Morales *et al.*, 2004). As well, CB_1 receptors have been shown to be expressed on 5-hydroxytryptaminergic neurons (Lau and Schloss, 2008) which suggests that one potential mechanism of the regulation of nausea by cannabinoid agonists is in the suppression of the release of 5-HT (Nakazi *et al.*, 2000). Indeed, 5-HT has been shown to evoke endocannabinoid release, which retrogradely suppresses excitatory synapses (Best and Regeher, 2008). Kwiatkowska *et al.* (2004) reported that combined sub-threshold low doses of Δ^9- THC and ondansetron (that individually were ineffective) completely suppressed cisplatin-induced vomiting in shrews, suggesting a synergistic effect. The potentiation of LiCl-induced nausea by AM251 could therefore be mediated by inverse agonism of the presynaptic CB_1 receptors on the terminals of 5-hydroxytryptaminergic neurons, thereby elevating the release of 5-HT in the insular cortex. Future studies that deliver AM251 directly to the insular cortex are needed to test this hypothesis.

Although central AM251 did not potentiate LiCl-induced conditioned gaping reactions in rats, it

did unconditionally suppress saccharin palatability when delivered to the lateral ventricle but not to the 4th ventricle, as was also seen with systemic AM251 in Experiment 1 (replicating Jarrett *et al.*, 2007). As icv administration to the 4th ventricle would be less likely to activate forebrain mechanisms than icv administration to the lateral ventricle (Blevins *et al.*, 2004; Fan *et al.*, 2004; Sink *et al.*, 2009b), the palatability shift produced by AM251 during conditioning, is most likely to be mediated by its action on forebrain mechanisms. Indeed, recent evidence suggests that direct administration of anandamide to the medial shell of the nucleus of the accumbens potently doubled the number of positive 'liking' facial reactions that sucrose taste elicits from rats (Mahler *et al.*, 2007). The unconditional attenuation of hedonic reactions to intraoral delivery of saccharin solution that was produced by AM251 when administered to the lateral ventricle or systemically may also reflect anhedonia, a depression-like effect in rats (Hill and Gorzalka, 2005). This anhedonia was not produced by systemic administration of the neutral CB_1 receptor antagonists, AM6545 or AM6527, even at doses as high as 8 $mg \cdot kg^{-1}$ (6 times higher than the effective systemic dose of AM251, 1.25 $mg \cdot kg^{-1}$). As the debilitating side effect of depression prevented the therapeutic use of the inverse agonist, rimonabant, in the treatment of obesity, the finding that the neutral CB_1 receptor antagonists did not attenuate hedonic reactions elicited by saccharin solution at doses that suppress feeding and weight gain (Sink *et al.*, 2008; Cluny *et al.*, 2010; Tam *et al.*, 2010), suggest that these compounds may have therapeutic potential.

When administered systemically or centrally to either the lateral ventricle or the 4th ventricle, AM251 also conditionally suppressed the palatability of saccharin solution on the subsequent drug-free test trial. This conditioned suppression of hedonic reactions, however, was not accompanied by a conditioned enhancement of gaping reactions. Such a conditional shift in hedonic reactions, does not appear to be a specific marker of nausea in rats, because even non-emetic treatments, such as amphetamine and cocaine, produce such a shift, as well as taste avoidance (Parker, 1995). As suppressed ingestive behaviour in the TR test follows a pattern to that of taste avoidance in a consumption test, it would be interesting to determine if central administration of AM251 would produce conditioned taste avoidance in rats, which may reflect conditioned fear rather than conditioned nausea in this non-emetic species (Parker *et al.*, 2008).

One of the primary therapeutic targets of CB_1 receptor antagonists is the suppression of feeding and food-motivated behaviours; however, the locus of action of these agents is controversial. CB_1 receptor agonists injected into the hypothalamic nuclei or into the nucleus accumbens have been shown to produce hyperphagia (Williams and Kirkham, 1999; Jamshidi and Talyor, 2001; Verty and Mallet, 2005; Soria-Gomez *et al.*, 2007) and these effects were blocked by CB_1 receptor inverse agonists. Although some investigators have reported that forebrain injections of CB_1 receptor inverse agonists produce anorexia (Werner and Koch, 2003; Verty *et al.*, 2004a,b), others have reported that feeding-related actions of these compounds may be peripherally mediated (Gomez *et al.*, 2002; Sink *et al.*, 2009b). Most recently, Sink *et al.* (2009b) reported no effect on feeding-related behaviours, of icv administration of AM251 to the lateral ventricles at doses up to 1/10 the peripherally effective dose. As CB_1 receptor inverse agonists produce nausea and depression in humans (see Janero and Makriyannis, 2009), there is considerable interest in the development of CB_1 receptor antagonists without central inverse agonist properties. Indeed, the CB_1 receptor specific neutral antagonist, AM6545, with limited CNS penetration dose-dependently reduced food intake and induced sustained reduction in body weight in mice and rats, and did not produce conditioned gaping at effective doses (Cluny *et al.*, 2010). The effect on food intake was maintained in rats with a complete subdiaphragmatic vagotomy. The results reported here, suggest that neutral CB_1 receptor antagonists, AM6545 and AM6527, neither enhanced the nauseating effects of LiCl, nor produced anhedonia in the TR test as did the inverse agonist AM251. The potentiation of LiCl-induced nausea by inverse agonism of the CB_1 receptor by AM251 may be peripherally mediated or centrally mediated by its action on CB_1 receptors distal to the cerebral ventricles.

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Conflicts of interest

There are no conflicts of interest among any authors.

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